IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 10/725,214 Confirmation No. 4443

Applicants : Muraleedharan G. Nair, Yanjun Zhang and Shaiju Vareed

Title : METHOD FOR INHIBITING CANCER CELLS

Filed : December 1, 2003

TC/A.U. : 1655

Examiner : Michele C. Flood

Docket No. : MSU 4.1-672

Customer No. : 21036

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SUPPLEMENTAL DECLARATION UNDER 37 C.F.R. § 1.132

Dear Sir:

Muraleedharan G. Nair states as a supplement to his Declaration Under 37 CFR 1.132 dated June 23, 2006 as follows:

(1.) That Cancer Letters 194, pages 13 to 19 (2003) (Exhibit A; attached), of which he is one of the authors, clearly shows that there is a direct correlation between *in vitro* and oral *in vivo* use in suppressing multiplicity of human cancer cells of the stomach or colon with anthocyanins and cyanidin. Malvidin is a related compound to cyanidin (see Figures 1 and 2 of the application). It would be expected by one skilled in the art that there would be a similar correlation with *in vitro* and *in vivo* use of malvidin to suppress multiplicity of stomach or colon cancer cells and the adenoma of new Claims 8 to 10.

(2.) That in his opinion, the results with malvidin *in vitro* are predictive of *in vivo* activity suppressing multiplicity of cancer cells based upon his research as set forth in the Cancer Letters publication.

(3.) That the undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements

were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Muraleedharan G. Nair

Date: 9-20-06

EXHIBIT A

Exhibit A

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Cancer Letters 194 (2003) 13–19
CANCER Letters
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Tart cherry anthocyanins inhibit tumor development in Apc^{Min} mice and reduce proliferation of human colon cancer cells☆

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Received 1 April 2002; received in revised form 23 September 2002; accepted 25 September 2002

Abstract

Anthocyanins, which are bioactive phytochemicals, are widely distributed in plants and especially enriched in tart cherries. Based on previous observations that tart cherry anthocyanins and their respective aglycone, cyanidin, can inhibit cyclooxygenase enzymes, we conducted experiments to test the potential of anthocyanins to inhibit intestinal tumor development in Apc^{Min} mice and growth of human colon cancer cell lines. Mice consuming the cherry diet, anthocyanins, or cyanidin had significantly fewer and smaller cecal adenomas than mice consuming the control diet or sulindac. Colonic tumor numbers and volume were not significantly influenced by treatment. Anthocyanins and cyanidin also reduced cell growth of human colon cancer cell lines HT 29 and HCT 116. The IC_{50} of anthocyanins and cyanidin was 780 and 63 μM for HT 29 cells, respectively and 285 and 85 μM for HCT 116 cells, respectively. These results suggest that tart cherry anthocyanins and cyanidin may reduce the risk of colon cancer.

Keywords: Anthocyanins; Cancer; Colon; Intestine; Mouse

1. Introduction

Tart cherries contain substantial quantities of anthocyanins in addition to other bioflavonoids [1]. Anthocyanins (Fig. 1), a member of the bioactive phytochemicals, are widely distributed in fruits, vegetables and beans, suggesting that plant-based diets can provide considerable amounts of anthocyanins [2,3]. Like the vast majority of flavonoids, anthocyanins primarily occur in plants as glycosides. Cyanidin is the major anthocyanin aglycone in tart cherries. Montmorency and Balaton™ tart cherries contain 0.40–0.80 mg/g, respectively, of anthocyanins [1]. These anthocyanins were found to function as antioxidants and cyanidin was shown to inhibit the activities of cyclooxygenase (COX) enzymes in vitro [2,4]. Several studies have demonstrated that non-

☆ Supported by USDA grant 99-35503-8147 (M.G.N., L.D.B.), the Michigan State University Project GREEEN Initiative, and the Cherry Marketing Institute.

Abbreviations: COX, cyclooxygenase; NSAIDs, non-steroidal anti-inflammatory drugs; APC, adenomatous polyposis coli; Min, multiple intestinal neoplasia; PBS, phosphate buffered saline; DMSO, dimethyl sulfoxide.

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doi:10.1016/S0304-3940(02)00583-9

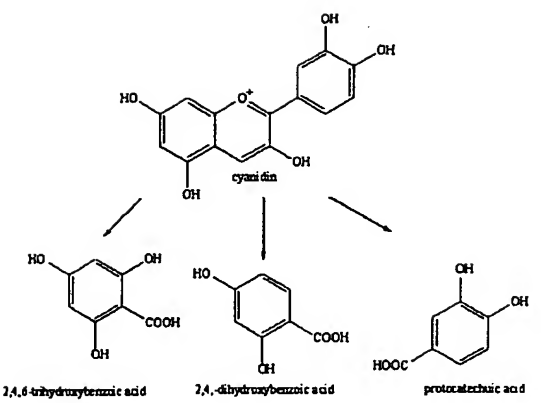

Fig. 1. Structures of the predominant tart cherry anthocyanin aglycone, cyanidin, and its major degradation products in cell culture medium.

steroidal anti-inflammatory drugs (NSAIDs) inhibit the growth of colon tumors in animal models and reduce the risk of colon cancer in humans [5,6]. In most cases, colon carcinogenesis depends on mutation of the adenomatous polyposis coli (APC) gene, which is considered a gatekeeper in the carcinogenic process [7]. Human APC gene germline mutations cause familial adenomatous polyposis, an autosomal dominantly inherited disease that predisposes affected individuals to develop numerous adenomatous polyps and, ultimately, colorectal cancer. APC gene mutations also are a frequent and early event in sporadic colon cancer. Apc^{Min} mice are a mutant mouse lineage predisposed to multiple intestinal neoplasia (Min) due to a mutation in the murine homolog of the APC gene [8]. The primary phenotype of Apc^{Min} mice is the development of multiple intestinal adenomas.

The objectives of this research were to determine the potential of tart cherry anthocyanins and cyanidin to inhibit intestinal tumor development in Apc^{Min} mice and to determine the potential of anthocyanins and cyanidin to directly inhibit the growth of human colon cancer cells.

2. Materials and methods

2.1. Animals and diets

This research was conducted with approval of the Michigan State University All-University Committee on Animal Use and Care. Apc^{Min} mice were produced by mating normal C57BL/6J ($Apc^{+/+}$) female mice with Min C57BL/6J ($Apc^{Min/+}$) male mice. Apc^{Min} progeny were identified by a polymerase chain reaction (PCR)-based assay [8] and were randomly assigned to five treatment groups ($n = 10$ per group; equal numbers of males and females) at 4–5 weeks of age and fed treatment diets for 10 weeks. The treatments were: (1) control diet (modified American Institute of Nutrition 93G diet containing 220 g/kg protein, 150 g/kg soybean oil and 50 g/kg cellulose); (2) control diet + 800 mg/l anthocyanins in the drinking water; (3) control diet + 200 mg/l cyanidin in the drinking water; (4) control diet + 200 mg/l sulindac (an NSAID) in the drinking water; and (5) modified control diet containing 200 g/kg freeze-dried pitted tart cherries. Cherries were frozen, freeze-dried and ground before they were incorporated into the diet. Anthocyanins were isolated from tart cherries and were a mixture of 3-cyanidin 2″-*O*-β-D-glucopyranosyl-6″-*O*-α-L-rhamnopyransyl-β-D-glucopyranoside [1] and 3-cyanidin 6″-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside [2] at 65 and 35%, respectively. The aglycone, cyanidin [3] (Fig. 1), was prepared from the anthocyanins [1]. Ascorbic acid (50 mg/l) was added to the drinking water of all mice to enhance the stability of anthocyanins and cyanidin in solution by lowering the pH. The level of anthocyanins and cyanidin used in this experiment were based on concentrations previously demonstrated to have antioxidant or COX-inhibitory activities in vitro. The diet containing tart cherries used in this study was not balanced to the other treatments on the basis of anthocyanin concentration, but rather represented the highest level of substitution of cherries into the control diet that could realistically be achieved without compromising the nutritional adequacy of the diet. Sulindac was included as a control in this experiment because it has consistently been shown to inhibit small intestinal adenoma development in Apc^{Min} mice.

2.2. Tumor number and size

The mice were sacrificed after 10 weeks of treatment and the numbers and sizes of adenomas in the intestinal sections were measured. The entire small intestine, cecum, and colon were removed from each mouse to determine the number and size of

adenomas. Intestinal sections were opened longitudinally, rinsed thoroughly with water, fixed overnight in 10% neutral-buffered formalin, and then stained with 0.2% methylene blue. Tumor numbers and dimensions for each intestinal segment were determined by direct counting with the aid of a dissecting microscope and measuring grid. The tumor sizes were determined by measuring the spherical (three dimensional) volume of adenomas in the cecum and colon and the average diameter of tumors in the small intestine. Tumors in the cecum and colon of Apc^{Min} mice typically are polypoid in appearance, whereas the small intestinal tumors are sessile. Spherical volumes of cecal and colonic tumors were calculated by the formula: Volume = 0.524 × (width × length × height of tumor).

Tumor numbers and diameters in the small intestine were analyzed by two-way analysis of variance (treatment, sex). For tumor numbers and volumes in cecum and colon, data were transformed to ranks and then ranks were analyzed by two-way analysis of variance. When significant treatment effects were detected ($P < 0.05$), treatment means were compared using the Least Significant Difference method. Six mice (one from the control group, two from the anthocyanin group, and three from the cyanidin group) were excluded from the final statistical analysis because they did not develop intestinal tumors. Confirmatory PCR analysis conducted at the end of the experiment indicated that these animals did not carry the APC^{Min} gene mutation.

2.3. Cell culture and growth assays

The human colorectal cancer cell lines HCT 116 and HT 29 (American Type Culture Collection) were cultivated in McCoy's 5A media supplemented with 10% fetal bovine serum. Cells were harvested for growth assays when they had reached 50–80% confluence by trypsin:ethylenediaminetetraacetic acid (EDTA) treatment and counted using a hemacytometer. Cells were then seeded at 15 000 cells/well in 24-well tissue culture plates. Plates were incubated overnight at 37°C and 5% CO_2 to allow cells to attach and begin proliferating.

At the beginning of treatments, the media was gently aspirated from each of the wells, which were then rinsed with phosphate buffered saline. One ml of treatment media was added to each well ($n = 8–12$ wells per treatment level per cell line) and plates were incubated for 72 h. Treatment media was McCoy's 5A media supplemented with 10% fetal bovine serum and containing the respective concentrations of anthocyanins (0–1000 μM) or cyanidin (0–250 μM). Anthocyanins were dissolved in distilled water before addition to the treatment media, whereas cyanidin was dissolved in dimethyl sulfoxide (DMSO) before addition. When used, the DMSO concentration was equalized for all treatment media and never exceeded 0.1% (v/v) of the final treatment media.

Total cell numbers in each well were quantified after 72 h of incubation in treatment media. Cell numbers were calculated based on total DNA content in each well using a procedure that quantifies DNA based on fluorescence of bound Hoescht 33258 [9]. Fluorescence was measured by a Cytofluor II fluorimeter (Applied Biosystems; Foster City, CA) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Fluorescence readings were converted to DNA by comparison to standard solutions of Salmon testis DNA (Sigma Chemical Company; St. Louis, MO). The cell

Table 1
Influence of anthocyanins, cyanidin, tart cherries and sulindac on cecal adenoma number and volume in Apc^{Min} mice (SEM = standard error of the mean)[a]

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	2.3[a]	0.6	3.0[a]	0.8
Anthocyanins	0.6[b]	0.6	0.7[b]	0.9
Cyanidin	0.6[b]	0.6	0.6[b]	1.0
Cherries	0.6[b]	0.5	1.8[b]	0.8
Sulindac	4.0[a]	0.5	4.0[a]	0.8

[a] [a,b]Means in the same column not sharing a common superscript a different ($P < 0.05$).

numbers in each well were calculated by converting the quantity of DNA in each well by the amount of DNA present in each cell (determined experimentally) for HCT 116 and HT 29 cells.

Cell numbers observed in each well after 72 h of growth were corrected for initial cell number (determined at the time treatment media was added). These data were then subjected to multiple regression analysis using the general linear models procedure of SAS (Version 8.1, SAS Institute, Inc., Cary, NC, USA) to develop least-squares polynomial equations describing the influence of anthocyanins or cyanidin concentration on cell number. These equations were then used to iteratively calculate the concentration of anthocyanins or cyanidin required to cause a 50% reduction (IC_{50}) in growth (cell number) for each cell line.

3. Results

Final body weights of mice were significantly influenced by treatment and averaged 22.8, 24.1, 21.3, 19.7, and 25.5 g for mice consuming control diet, anthocyanins, cyanidin, tart cherries, and sulindac, respectively. Final body weights for mice consuming anthocyanins and sulindac were greater ($P < 0.05$) than for mice consuming tart cherries.

Treatments had differential effects on tumor incidence and burden in the various sections of the intestinal tract. Mice consuming anthocyanins, cyanidin, or tart cherries had fewer ($P < 0.05$) adenomas in the cecum than mice consuming the control diet or sulindac (Table 1). The total burden (volume) of cecal adenomas was less ($P < 0.05$) in mice consuming anthocyanins, cyanidin or tart cherries when compared to mice consuming the control diet or sulindac (Table 1). Colonic adenoma number was not significantly influenced by treatment (Table 2). Although mice that consumed tart cherries had the greatest adenoma burden in the colon (8.4 mm^3), this was not statistically greater than that observed in mice consuming the other treatments (Table 2).

Tumor multiplicity in the small intestine was not significantly influenced by treatment and averaged 48 tumors per mouse (Table 3). Mice that consumed sulindac had the smallest number of small intestinal adenomas (28 per mouse), but this was not statistically different than small intestinal tumor numbers observed for the other treatments. The average size of small intestinal adenomas (Table 3) was increased ($P < 0.05$) by feeding tart cherries and reduced ($P < 0.05$) by sulindac relative to that observed in mice consuming the control diet, anthocyanins, or cyanidin.

Treatment with anthocyanins (Fig. 2) or cyanidin (Fig. 3) caused a dose-dependent reduction in cell numbers for both HCT 116 and HT 29 cells. Neither anthocyanins nor cyanidin caused cytotoxicity even at the highest concentrations tested, as indicated by little or no dead cells. Cyanidin was far more effective in inhibiting the growth of these cancer cell lines than anthocyanins. The observed IC_{50} values for cyanidin were 85 and 63 μM for HCT 116 and HT 29 cells, respectively, whereas those for anthocyanins were 260 and 585 μM for HCT 116 and HT 29 cells, respectively.

4. Discussion

Our interest in testing the potential of tart cherry anthocyanins and cyanidin to inhibit tumor development in Apc^{Min} mice stemmed from the observation that these compounds inhibit the activities of COX

Table 2
Influence of anthocyanins, cyanidin, tart cherries and sulindac on colon adenoma number and volume in Apc^{Min} mice (SEM = standard error of the mean)

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	3.7	0.7	2.4	2.0
Anthocyanins	3.1	0.7	4.0	2.1
Cyanidin	3.7	0.8	3.5	2.3
Cherries	3.3	0.6	8.4	1.9
Sulindac	5.3	0.6	3.3	1.9

Table 3
Influence of anthocyanins, cyanidin, tart cherries and sulindac on small intestinal adenoma number and diameter in Apc^{Min} mice (SEM = standard error of the mean)[a]

Treatment	Adenomas/mouse	SEM	Adenoma volume/mouse (mm^3)	SEM
Control	58	13	1.39[a]	0.10
Anthocyanins	76	13	1.34[a]	0.10
Cyanidin	54	16	1.25[a]	0.11
Cherries	42	12	1.66[b]	0.10
Sulindac	28	12	0.93[c]	0.09

[a] [a,b,c]Means in the same column not sharing a common superscript a different ($P < 0.05$).

enzymes [4]. Other studies have demonstrated that sulindac (and other NSAIDs) reduce small intestinal tumor multiplicity and size in Apc^{Min} mice [10–12]. In this study, we found that anthocyanins, cyanidin, and tart cherries (presumably as a source of anthocyanins) all significantly reduced the number and burden of tumors in the cecum of Apc^{Min} mice. Conversely, sulindac did not influence tumor development in the cecum. None of the treatments tested influenced the numbers of tumors in the small intestine or the numbers or burden of tumors in the colon. Our inability to detect a significant reduction in small intestinal adenoma number by sulindac was likely due to a number of factors, including the relatively small numbers of mice per treatment group and large variations among individual mice in adenoma development. The dose of sulindac and duration of tumor promotion allowed in this experiment may also have contributed to the limited effect of sulindac on small intestinal adenoma number. The lack of effect of anthocyanins or cyanidin on colonic tumor development may be a consequence of their metabolism by intestinal bacteria or their spontaneous degradation in the cecal and colonic environment.

The average size of small intestinal tumors was smaller in mice consuming sulindac and larger in mice consuming tart cherries when compared to that in mice consuming the control diet, anthocyanins or cyanidin. Progression in the size of small intestinal tumors is highly correlated with morbidity in Apc^{Min} mice. We have observed that significant morbidity and weight loss occurs when small intestinal tumors reach an average size of 1.5 mm. At this stage, the tumors tend to hemorrhage and may perforate the small intestine. The observed differences in final body weight in this experiment are a consequence of these differences in small intestinal tumor promotion. It is well documented that sulindac and other NSAIDs consistently reduce the size of small intestinal tumors in Apc^{Min} mice [10–12]. The mechanism for the larger small intestinal tumor size in mice consuming tart cherries is not known, but merits further investigation.

The effects of the treatments on tumor development were not consistent throughout the intestinal

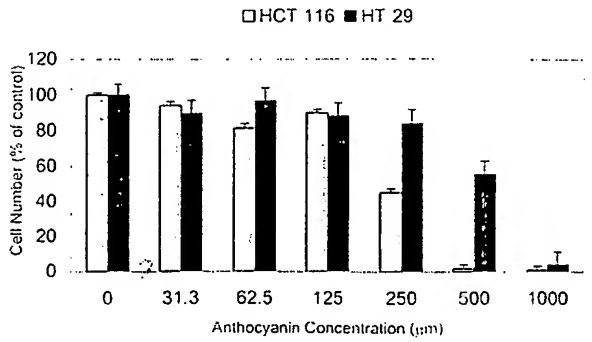

Fig. 2. Influence of anthocyanins on growth of human colon cancer cells. Gray bars, HCT 116 cells; Black bars, HT 29 cells. Error bars = standard error of the mean.

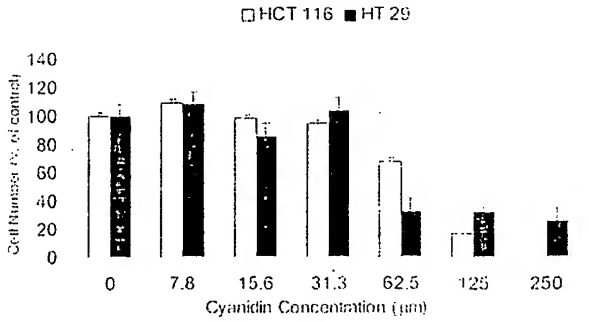

Fig. 3. Influence of cyanidin on growth of human colon cancer cells. Gray bars, HCT 116 cells; Black bars, HT 29 cells. Error bars = standard error of the mean.

tract. Sulindac and other NSAIDs typically reduce small intestinal tumor development in Apc^{Min} mice, but often have little effect on tumor development in the cecum and colon [13]. Mice consuming anthocyanins, cyanidin and tart cherries had fewer and smaller tumors in the cecum, but these compounds did not afford any protection to the small intestine. These results indicate that although anthocyanins and cyanidin inhibit the activities of COX enzymes in vitro [2,4], they likely do not influence intestinal tumor development in Apc^{Min} mice through a pathway involving COX inhibition. Meiers et al. [14] demonstrated that the anthocyanins cyanidin and delphinidin are potent inhibitors of the epidermal growth factor receptor kinase. Administration of inhibitors to the epidermal growth factor receptor kinase also has been shown to reduce intestinal adenoma development in Apc^{Min} mice [15]. We currently are investigating other potential mechanisms whereby anthocyanins and cyanidin influence intestinal tumor development.

Both anthocyanins and cyanidin inhibited the growth of the colon cancer cell lines HT-29 and HCT 116, although cyanidin was much more effective. Our observations are in agreement with those of Meiers et al. [14], who observed IC_{50} values 73 and 42 μM when cyanidin was administered to LXFL529L large cell lung tumor cells and A431 human vulva carcinoma cells, respectively. Kamei et al. [16] also demonstrated that a crude anthocyanin fraction prepared from red wine was an effective inhibitor of the growth of HCT-15 human colon tumor cells in vitro. We have identified three degradation products from anthocyanins and cyanidin in cell culture medium. These were protocatechuic acid, 2,4-dihydroxybenzoic, and 2,4,6-trihydroxybenzoic acids [3]. In addition, we have detected trace quantities of cyanidin-3-glucoside and cyanidin in culture medium after 72 h of cell growth in anthocyanin treatments. We have evaluated the potential of these degradation products to inhibit the growth of HCT 116 and HT 29 cells. None of the degradation compounds assayed demonstrated any inhibition of cell growth at concentrations ranging up to 250 μM [3].

Anthocyanins and cyanidin are unstable at pH 7.0 and spontaneously degrade to chalcone and benzoic acid derivatives. The red cyanidin cation is stable at pH <3, but deprotonates and produces ketoquinonoidal bases and finally to an ionized quinonoid base at pH >7 [3]. At pH 3–6, the cyanidin cation forms a carbinol pseudobase or chalcone pseudobase [3].

Anthocyanins are highly water-soluble and considered to be structurally similar to a number of strong DNA intercalators [17]. Both DNA and RNA act as strong copigments for anthocyanins [18]. Also, anthocyanins protect DNA against oxidative damage [19]. Under in vivo and cell culture conditions, both anthocyanins and cyanidin potentially form corresponding pseudobases due to pH variations. These pseudobases are transition compounds and may be stable in vivo as protein bound complexes. Given these results, we predict that the anthocyanins, the aglycone cyanidin, or its varying pseudobases directly suppress cell growth and subsequent tumor development.

We believe that our results are the first to demonstrate that anthocyanins and cyanidin have the potential to directly interfere with intestinal tumor development. Hagiwara et al. [20] demonstrated that anthocyanins in purple corn color reduced the promotion of colon tumors caused by 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) in rats initiated with dimethyl hydrazine. However, they did not test the potential of purple corn color anthocyanins to directly suppress tumor development [20].

Although we did not directly measure intake of anthocyanins or cyanidin in this experiment, we can tentatively compare the doses administered to the mice to a comparable human dose. If we assume that the mice consumed 8 ml of water each day and also assume that there was no degradation of anthocyanins or cyanidin, then the consumed dose would be 6.4 or 1.6 mg of anthocyanins or cyanidin per day, respectively, for the mice consuming those treatments. If we assume an average weight of 25 g for the mice and 70 kg for an adult human, then the comparative human doses (on the basis of $kg^{0.75}$) would be 2400 or 600 mg of anthocyanins or cyanidin, respectively. It is very unlikely that these doses could be achieved in a typical human diet without supplementation. It should also be noted that these calculations represent very crude comparisons and do not consider potential absorption, metabolism or degradation of the compounds.

In summary, we have demonstrated that tart cherry anthocyanins and their aglycone cyanidin significantly reduced tumor development in the cecum of

Apc^{Min} mice. These compounds also directly inhibited the growth of human colon cancer cells in vitro, with the aglycone cyanidin being far more effective than the anthocyanin glycosides. Benzoic acid derivatives yielded from the degradation of anthocyanins and cyanidin have no influence on colon cancer cell growth. Taken together, these results suggest that and cyanidin or its corresponding pseudobase is directly inhibiting tumor development in the cecum of Apc^{Min} mice. Anthocyanins also are effective presumably due to their deglycosylation to cyanidin by cecal bacteria. The lack of a clear suppression of tumor development in the colon probably is due to further degradation of the cyanidin molecule by elevated pH in the intestinal lumen and bacterial metabolism.

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